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Abstract

There are currently no treatment methods on the market for modifying OA – only drugs that help relieve pain and inflammation but do not address the underlying disease, allowing it to continue destroying joint tissues. When joint function becomes severely impaired and other management strategies are ineffective, arthroscopic or joint replacement surgeries become the only options for patients. More than 50% of people worldwide who are over 65 years of age show radiological evidence of OA. These patients and younger populations who are susceptible to OA onset due to sports injuries or other factors could significantly benefit from the development of strategies to mitigate disease progression. Furthermore, post-traumatic OA experienced by military personnel injured on the battlefield is a highly accelerated process, likely because these combat injuries are complicated by factors that are associated with greater risk including bone loss, surrounding soft tissue damage, and infection. In these combat-related cases, post-traumatic OA typically manifests less than 2 years after injury compared to post-traumatic OA resulting from sports injuries that takes about 10 years to develop.

The goals of this work are to test the ability of a novel therapeutic to slow the progression of post-traumatic osteoarthritis. This debilitating joint condition more severely affects military service personnel who have sustained injuries in combat resulting from high energy impacts such as explosions, fragment projectiles, and gunshot wounds. The therapeutic we propose is derived from human amniotic membrane, which has shown promise in clinical studies for various regenerative applications. The objectives of the work are to characterize two formulations of the injectable therapeutic, determine its capacity for treating OA in a small animal model, then scale up the approach to a clinically relevant loading and disease progression timeframe in a large animal model in order to outline a pathway to human clinical trials of the treatment method.

Our central hypotheses are that joint retention and therapeutic efficacy will be influenced by amniotic membrane particle size, treatment timing, and frequency of delivery in well-established small and large animal models of post-traumatic OA. These hypotheses will be tested via three Specific Aims: *Aim 1:* Evaluate the effects of human amniotic membrane (AM) particle size distribution on particle retention and progression of OA after 3 weeks in the rat medial meniscal transection (MMT) model. *Aim 2:* Assess differences in therapeutic efficacy of single and multiple post-injury particle injection treatments on OA progression during a 6 week time period. *Aim 3:* Evaluate therapeutic effects in an established ovine unilateral medial meniscectomy (MM) model of OA.

Table of Contents

1. INTRODUCTION:

Osteoarthritis (OA) is a debilitating articular joint condition resulting in functional impairment for nearly 27 million Americans at a cost of over $128 billion per year for the U.S. economy. Post-traumatic OA (PTOA) associated with combat extremity injuries has a 26% greater incidence in active military service personnel 20-24 years of age and over twice the incidence after age 40 compared to the general population. There are no disease modifying OA therapeutics currently approved. The goal of this work is to develop a safe and effective long-term strategy, using intra-articular delivery of micronized dehydrated amnion/chorion membrane (dHACM), to inhibit OA disease progression following trauma. Factors we hypothesized would impact therapeutic efficacy of dHACM included particle size, timing of treatment, and frequency of delivery. Thus, the study aims involved varying and more fully characterizing particle size distribution, injecting at different time points, and utilizing single or multiple injections, in both the rat medial meniscus transection (MMT) model and the sheep medial meniscectomy (MM) model.

2. KEYWORDS:

Osteoarthritis therapeutic	Intra-articular injection	Amnion/chorion membrane	Rat medial meniscal transection	Sheep medial meniscectomy
Particle size distribution	therapeutic timepoints	EPIC-μCT	Articular cartilage	Subchondral bone
Osteophytes	Proteoglycans			

3. OVERALL PROJECT SUMMARY:

In the third annual funding period (Sept 2016 – Sept 2017), significant progress was made in Specific Aims 1, 2, and 3; Major Tasks 1, 2, 3, 4, and 5.

***Specific Aim 1*:** Evaluate effects of micronized human amniotic membrane particle size distribution on particle retention and progression of OA after 3 weeks in the rat medial meniscal transection model

Major Task 1: Characterization of particle size distribution and retention.

Subtask 1: Characterize size distribution. (Completed and previously documented in Year 2 annual report).

Subtask 2: Submit documents for IACUC approval. (Completed and previously documented in Year 1 annual report).

Milestone #1: IACUC approval was received and previously documented in Year 1 annual report.

Subtask 3: Particle preparation and surgical procedures. (Completed and previously documented in Year 2 annual report).

Subtask 4: Establish particle retention profiles for two size distributions. (Completed and previously documented in Year 2 annual report).

Major Task 2: Assess 3 week therapeutic efficacy *in vivo* for two size distributions.

Subtask 1: Surgical and treatment procedures. (Completed and previously documented in Year 2 annual report).

Subtask 2: Inflammatory response evaluation. See Subtask 4.

Subtask 3: Evaluation of therapeutic effects. Therapeutic effects of dHACM at two particle size distributions (standard and reduced particle size (RPS)) was evaluated at 1 week and 3 weeks post-MMT after intra-articular treatment at 24 hrs post-MMT. (Completed and previously documented in Years 1 and 2 annual reports; 1 manuscript in press and 1 manuscript in preparation).

Additional investigation using pooled donor dHACM was performed in Year 3 with therapeutic efficacy assessed at 1 and 3 weeks post-MMT. Results indicated that therapeutic efficacy does depend on dHACM particle size with the standard (larger) being more effective. Highlights of these results are shown in Figures 1 & 2.

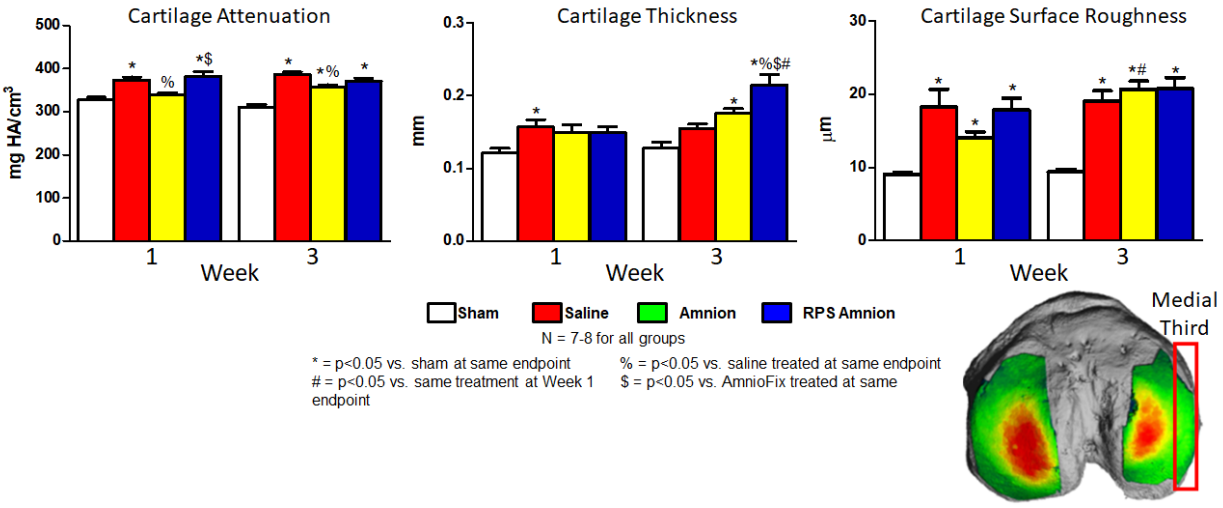

Figure 1. Cartilage morphology and composition were measured with EPIC-µCT in the medial, central, and lateral thirds of the medial tibial plateau at 1 and 3 weeks post-MMT. These plots show medial third quantifications for cartilage attenuation, thickness and surface roughness changes following treatment with standard particle size (Amnion) and reduced particle size (RPS Amnion) dHACM.

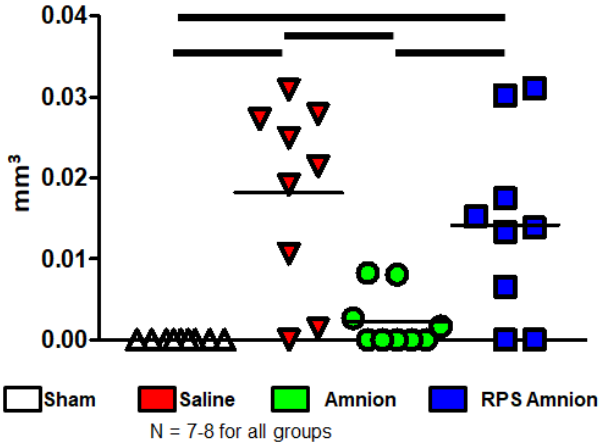

Figure 2. Quantification of lesion volume across the full medial tibial plateau was also performed for standard particle size (Amnion) and RPS dHACM (RPS Amnion) treatment 3 weeks after MMT surgery.

Reducing particle size did not further attenuate OA progression compared to standard particle size and in fact resulted in increased cartilage thickness and increased lesion volume at 3 weeks post-MMT.

Subtask 4: Synovial cellular response and particle retention.

Particle retention was quantified post-MMT surgeries via injection of two size distributions of dHACM labeled with a fluorophore and quantification of fluorescent efficiency over a duration of 42 days. Fluorescent efficiency was not significantly different at any timepoint when comparing the standard (µ-dHACM) and reduced particle size (RPS µ-dHACM) distributions (Figure 3), suggesting similar joint clearance times for the two particle size distributions.

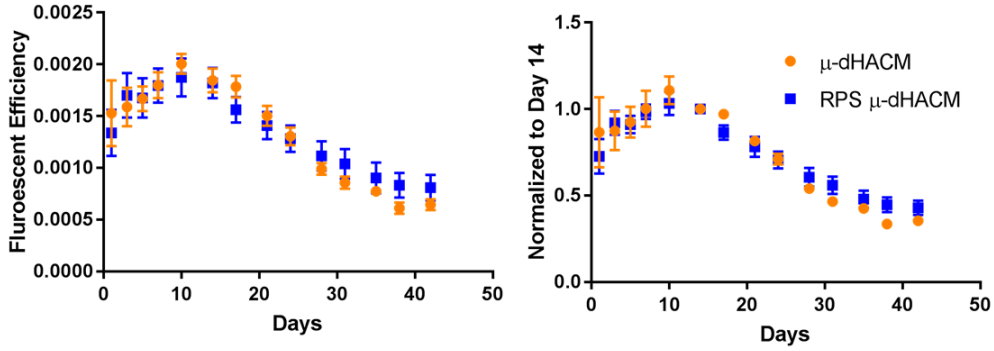

Figure 3. Quantification of fluorescent efficiency at time points out to 42 days for standard size distribution µ-dHACM and RPS µ-dHACM (fluorophore: VivoTag 680XL, Perkin Elmer IVIS Spectrum imaging). No significant differences between groups were detected (repeated measures ANOVA with Sidak multiple comparisons test). Quantification of saturated area demonstrated similar results (not shown).

In our work with the rat MMT model, histology showed dHACM particles embedded in the synovium and surrounded by cells. We thus hypothesized that the synovium and its cells play an important role in the attenuation of OA progression through dHACM-mediated release of proteases and inflammatory cytokines. While initially we had intended to extract synovial fluid from joints to assess inflammatory response of knee tissues to dHACM, this proved extremely challenging. The methodology for assessing synovial cellular response / inflammatory response (Subtasks 2 and 4) was therefore revised from the original proposal to an in vitro approach in two parts.

For the first experiment, human synoviocytes were grouped as either activated (exposed to media containing IL-1β and TNF-α to induce an inflammatory state, red in Figure 4) or inactivated (exposed to control media, blue in Figure 4). Both particle size distributions of dHACM were used, and the cells (via Transwell inserts) were exposed to either dHACM particles themselves or conditioned media that had been exposed to dHACM (labeled 'Extract' in Figure 4).

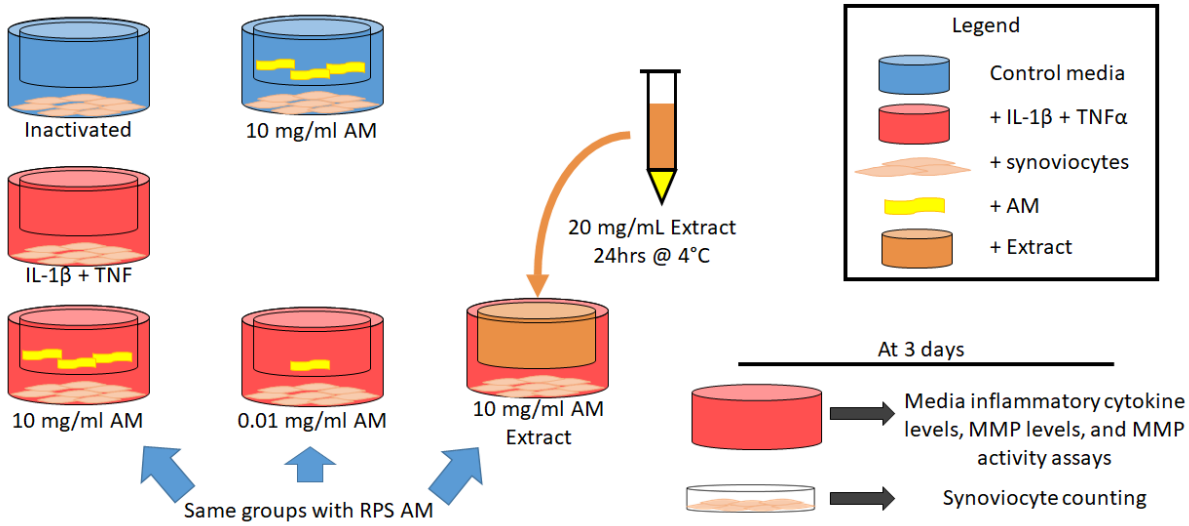

Figure 4. Experimental design schematic for evaluation of synoviocyte interactions with dHACM at two particle size distributions (standard and RPS, labeled AM) and two dose levels (0.01 and 10 mg/ml), with inflammatory state induced in culture by activation with IL-1β and TNF-α (red). Culture in control media was also included (inactivated synoviocytes).

Media inflammatory cytokine and MMP levels were quantified after 3 days, and a partial least squares discriminant analysis (PLS-DA) was performed. This analysis revealed group clustering and separation patterns that allowed for a more targeted assessment of the following two questions:

- How does dHACM affect activated and inactivated synoviocytes?
- How does changing particle size distribution and method of exposure alter synoviocyte response?

Some of the individual cytokine expression analyses, when comparing the effects on activated (media with IL-1β and TNF-α) or inactivated (control media) synoviocytes, are shown in Figure 5. IL-6 expression was higher for activated compared to inactivated synoviocytes, and treatment with dHACM elevated IL-6 expression compared to untreated. With dHACM treatment, MMP3 expression level was higher for activated compared to inactivated synoviocyte cultures. These markers are typically elevated for OA affected tissues. MCP-1 is a protein that modulates monocyte/macrophage recruitment, inflammation, and chondrocyte and cartilage degradation. Activated synoviocytes not treated with dHACM had elevated MCP-1 expression compared to inactivated untreated synoviocyte cultures. Treatment with dHACM significantly reduced the MCP-1 expression levels for activated synoviocyte cultures.

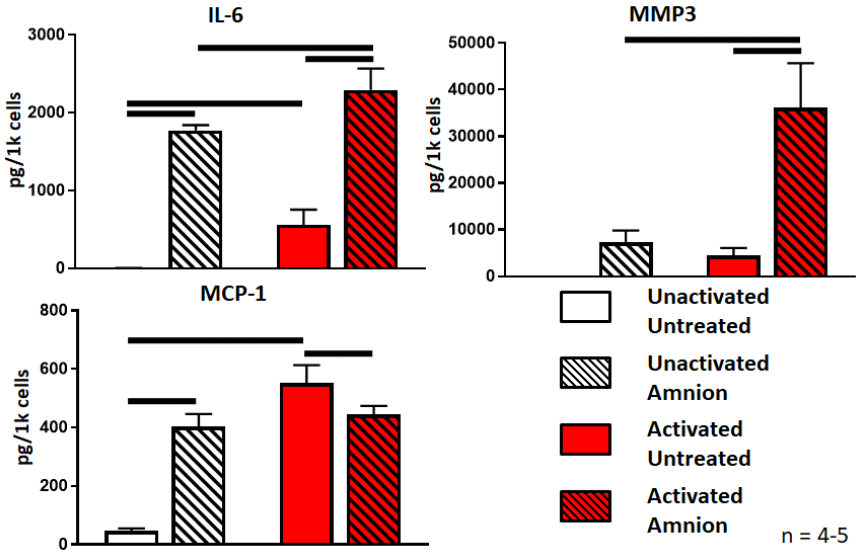

Figure 5. Effect of activation and dHACM treatment on IL-6, MMP3, and MCP-1 cytokine expression in synoviocyte cultures.

Effects of particle size distribution and dHACM exposure method on synoviocyte cultures were also further investigated, and Figure 6 shows a few examples of cytokine expression analyses. IL-6 expression was significantly lower for cultures treated with dHACM extract compared to Transwell exposure (for both particle size distribution of dHACM). Cultures treated with reduced particle size extract media increased IL-6 and MMP3 expression levels compared to the standard particle size distribution. MCP-1 levels were decreased for cultures treated with extract media from standard size dHACM particles compared to Transwell cultures. Treatment with RPS dHACM extract media elevated MCP-1 levels compared to cultures treated with standard dHACM particle size extract media.

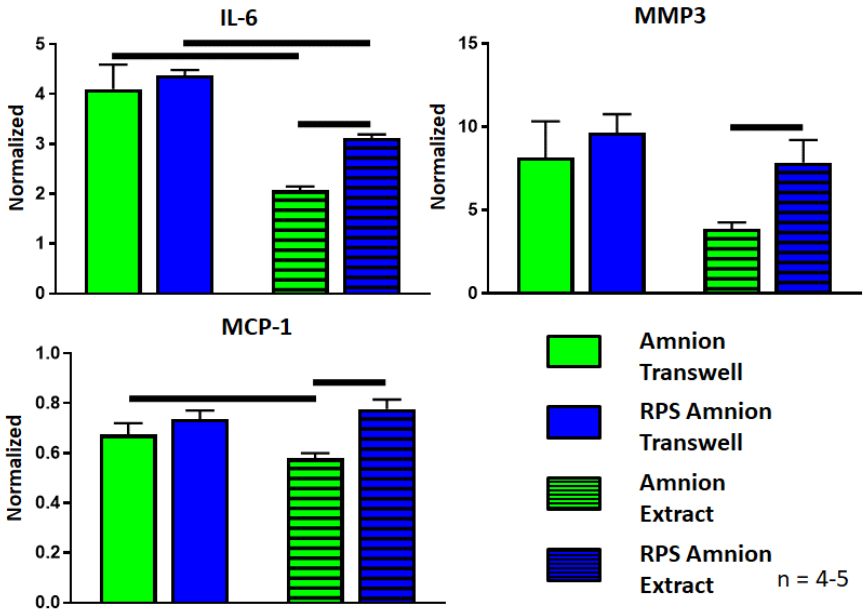

Figure 6. Effect of particle size distribution and exposure method on IL-6, MMP3, and MCP-1 cytokine expression in synoviocyte cultures.

For the second experiment, to further elucidate the influence of dHACM and synoviocyte/dHACM interactions on chondrocytes, a bovine chondrocyte pellet / human synoviocyte Transwell co-culture experiment was performed (Figure 7).

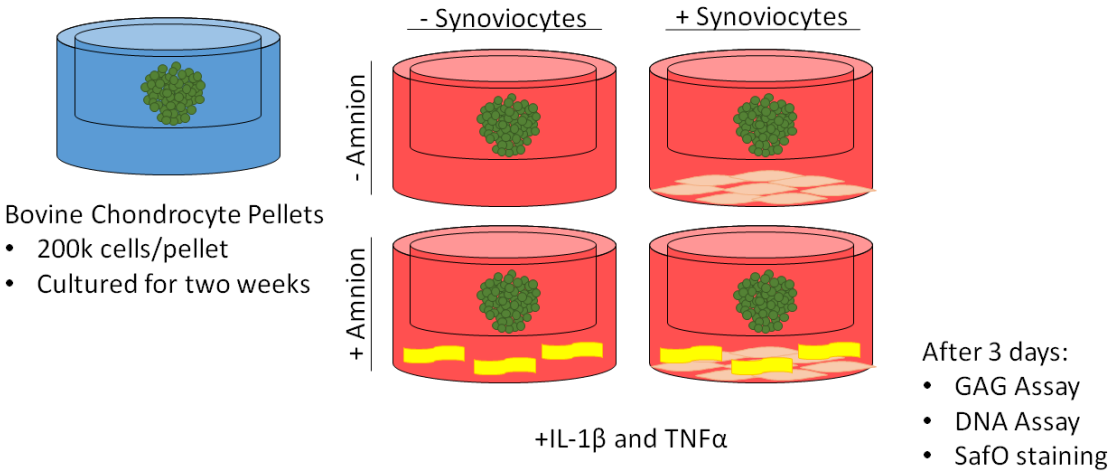

Figure 7. Schematic outlining experimental groups for investigating interactions between dHACM, synoviocytes, and chondrocytes in Transwell culture. Bovine chondrocytes in 200,000 cell pellets were cultured for two weeks, and Transwell culture of the pellets in 4 groups was continued for 3 days. IL-1β and TNF-α were added at 5ng/ml each. dHACM was added directly to the synoviocytes while pellets were kept separate via Transwell inserts. After 3 days, pellets were either digested for GAG and DNA analysis or processed for histology and safranin-O staining.

GAG results showed that addition of dHACM prevented cytokine- and synoviocyte-induced PG loss in chondrocyte pellets (Figure 8).

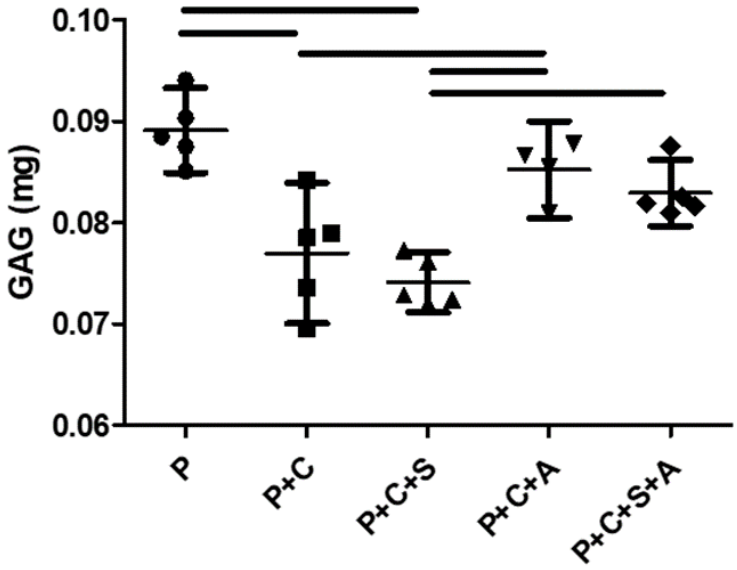

Figure 8. Quantification of GAG content from pellets from the 5 groups, where: P = pellet only, C = cytokines IL-1β and TNF-α, S = synoviocytes, A = dHACM.

Specific Aim 1 Conclusions

dHACM was successfully labeled with two fluorophores, and VivoTag 680 XL (Perkin Elmer) was chosen to perform further characterization of *in vivo* dHACM retention and localization in our studies of the rat MMT model of PTOA. Fluorescent efficiency quantification out to 42 days suggested no significant differences in clearance of dHACM and RPS dHACM particles in the treatment of rat knee joint degeneration induced by MMT.

Changes in articular cartilage and subchondral bone morphology and composition due to MMT surgery were able to be quantified at both 1 and 3 weeks after joint destabilization. Treatment with micronized dHACM demonstrated better preservation of cartilage proteoglycan (PG) content (inversely related to cartilage attenuation) at 1 and 3 weeks post-MMT. Reducing the particle size distribution did not further attenuate OA disease progression compared to the standard size distribution and resulted in increased cartilage thickness and lesion volume at 3 weeks. The majority of alterations to subchondral bone (density, thickness) were detected at 3 weeks (not at 1 week post-MMT) whereas cartilage attenuation and surface roughness differences were already seen at 1 week post-MMT. This suggests that in the rat MMT model, degenerative changes occur first in the articular cartilage and subchondral bone changes follow.

dHACM protected against cytokine- and synoviocyte-induced proteoglycan loss in the co-culture study. Generally speaking, cytokine/protease production by synoviocytes was

increased with exposure to dHACM, and further study is required to more fully understand the role of various factors on degenerative changes due to OA and the role of dHACM in modulating those effects to protect against degeneration.

Milestone #2: One manuscript, entitled, "Contrast Enhanced MicroCT Imaging of Early Articular Changes in a Pre-Clinical Model of Osteoarthritis," has been accepted for publication. Another manuscript addressing specific particle size distribution effects on therapeutic efficacy is currently in preparation. A third manuscript addressing the effects of dHACM (directly and with media extracts of dHACM) on human synoviocytes and the effects of dHACM on chondrocyte/synoviocyte co-cultures is in preparation.

***Specific Aim 2*:** Assess differences in therapeutic efficacy of single and multiple post-injury particle injection treatments on OA progression during a 6 week time period.

Major Task 3: Assess therapeutic efficacy for single or multiple intra-articular injections.

Subtask 1: Surgical and treatment procedures. (Completed and previously documented in Year 1 annual report)

Subtask 2: Inflammatory response evaluation. See Specific Aim 1, Subtasks 2 and 4.

Subtask 3: Evaluation of therapeutic effects using EPIC-μCT and histology. (Completed and previously documented in Year 1 annual report).

Subtask 4: Synovial cellular response and particle size retention. See Specific Aim 1, Subtasks 2 and 4.

Specific Aim 2 Conclusions

Articular cartilage composition, subchondral bone, and osteophyte data showed a beneficial effect of single dHACM injection at 3 wks post-MMT surgery when evaluated 3 wks after injection suggesting that after disease progression has begun, dHACM can provide protection against degradative effects. However, a single injection of dHACM 24 hrs post-MMT surgery did not provide a protective effect out to 6 wks post-surgery. Multiple injection dHACM treatment (at 24 hrs + 3 wks post-MMT surgery) demonstrated histological results suggesting moderate inhibition of cartilage damage; however, EPIC-μCT quantification showed somewhat inconclusive results.

Milestone #3: A manuscript, entitled, "Intra-Articular Delivery of Micronized Dehydrated Human Amnion/Chorion Membrane Reduces Degenerative Changes in Osteoarthritic Joints," including work summarized for Major Task 3, has been submitted for publication.

***Specific Aim 3*:** Evaluate therapeutic effects in an established ovine unilateral medial meniscectomy model of OA

Major Task 4: Produce and characterize ovine unilateral medial meniscectomy OA model.

Subtask 1: Submit documents for IACUC/ACURO and CRADA approvals. (Completed and previously documented in Year 2 annual report).

Subtask 2: Surgical procedures for ovine medial meniscectomy (MM). (Completed and previously documented in Years 1 and 2 annual reports)

Subtask 3: Ovine model EPIC-µCT protocol development. Initial protocol development was completed and previously documented in Year 1 annual report. However, because the original anionic contrast agent was discontinued, there was a need to explore replacement contrast agents and validate their usefulness in the rat MMT and ovine MM models for quantifying degenerative changes and dHACM therapeutic effects.

A cationic contrast agent, CA4+, was acquired in 3 iodine concentrations (8, 12, and 24 mgI/ml). An anionic agent, Conray™, was also acquired for testing. Proximal tibiae from naïve rats were immersed in contrast agent solution for cumulative times of 0, 10, 20, 30, 60, 90, and 180 mins (for Conray™ 0, 10, 20, 30, and 60 mins). At each timepoint, specimens were scanned and a histogram of X-ray attenuation values of the greyscale slices in the most proximal ~1.6mm was generated. Mean X-ray attenuation of the medial tibial plateau only was recorded to generate equilibration curves for these volumes of interest (VOIs).

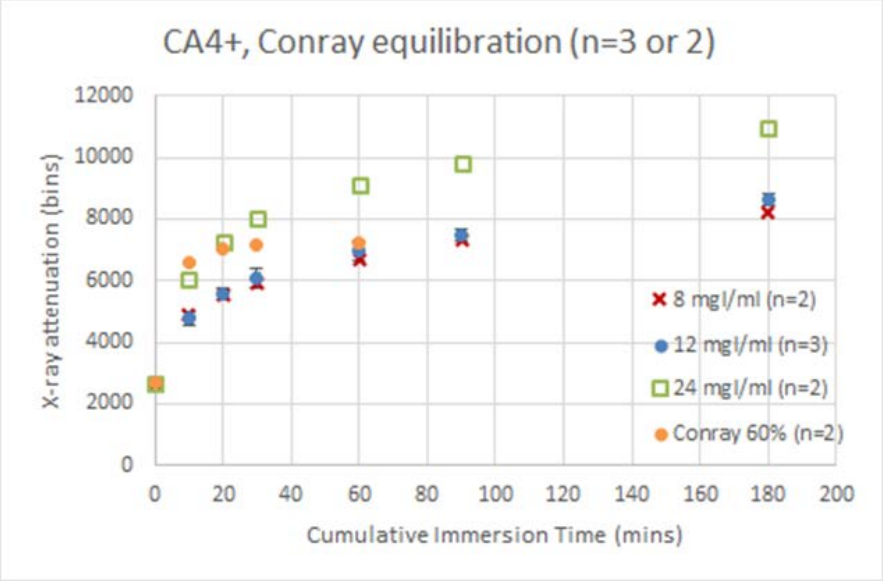

Figure 9. Average articular cartilage X-ray attenuation curves from CA4+ cumulative immersion times of 0, 10, 20, 30, 60, 90, 180 minutes for 8, 12, and 24 mgI/ml concentrations and Conray™ cumulative immersion times of 0, 10, 20, 30, 60 minutes for 60% solution in PBS (without calcium and magnesium). Error bars indicate standard deviations.

For CA4+, the 12 mgI/ml concentration showed the most consistent promise for further testing in MMT rat tibiae and ovine tissue, qualitatively by examining the 2D tomograms as well as when considering the histogram and equilibration analyses. 60% Conray™ also provided average X-ray attenuation values for cartilage in a range that was within that of the three CA4+ concentrations, but histogram analyses suggested more overlap at this concentration between cartilage and bone peaks compared to the CA4+ histograms. This suggested that a lower concentration may be more useful for continued articular cartilage testing.

The CA4+ X-ray attenuation curves also suggested a continued gradual increase in X-ray attenuation in cartilage tissue with increased cumulative immersion times, stressing the importance of maintaining a precise immersion time across all specimens. In contrast, Conray™, as confirmed by histogram comparisons, did not exhibit this effect (data not shown). For CA4+ and Conray™, 25 minutes immersion time was chosen for analyses of degenerated tissues post-MMT surgery.

MMT surgeries on left knees (right knees unoperated) were performed on 12 rats (n=3 per group) to conduct further contrast agent pilot testing on non-healthy joints. Animals were euthanized at 3 weeks post-MMT surgery, and all limbs fixed in 10% NBF prior to dissection and imaging. After joints are carefully dissected and proximal tibiae freed of extraneous soft tissue, they were immersed in contrast agent solutions for 25 minutes (8 and 12 mgI/ml CA4+ and 50% Conray™) or 30 minutes (Hexabrix™ – the original protocol immersion time) and evaluated using microCT.

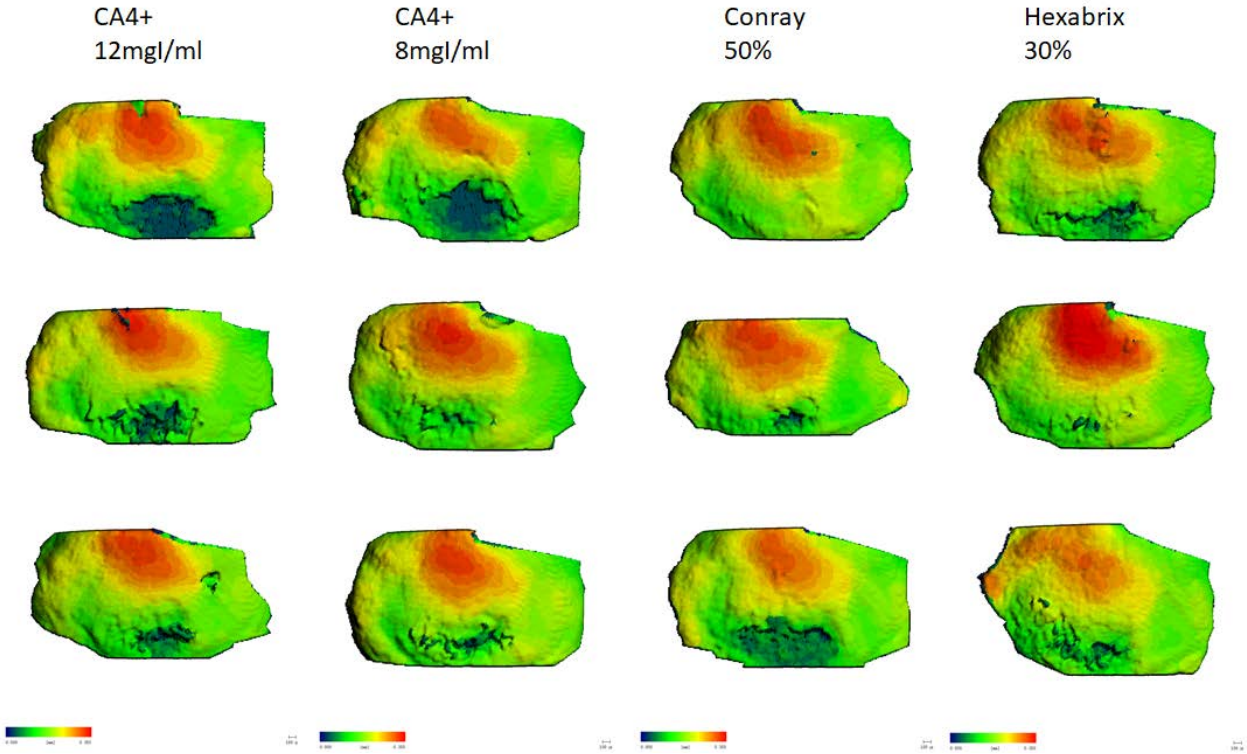

Figure 10. Medial tibial plateau articular cartilage thickness maps of left MMT joints, 3 weeks post-surgery. Cartilage contrast was enhanced with CA4+ at two concentrations (8

and 12 mgI/ml), Conray™ at 50%, and Hexabrix™ at 30% (original protocol concentration).

When qualitatively comparing Figure 10 images to the contralateral right knee images in Figure 11, degenerative changes are clearly visible on the most medial portion of the medial tibial plateau. This demonstrated that morphology changes would likely be quantifiable using any of the contrast agents.

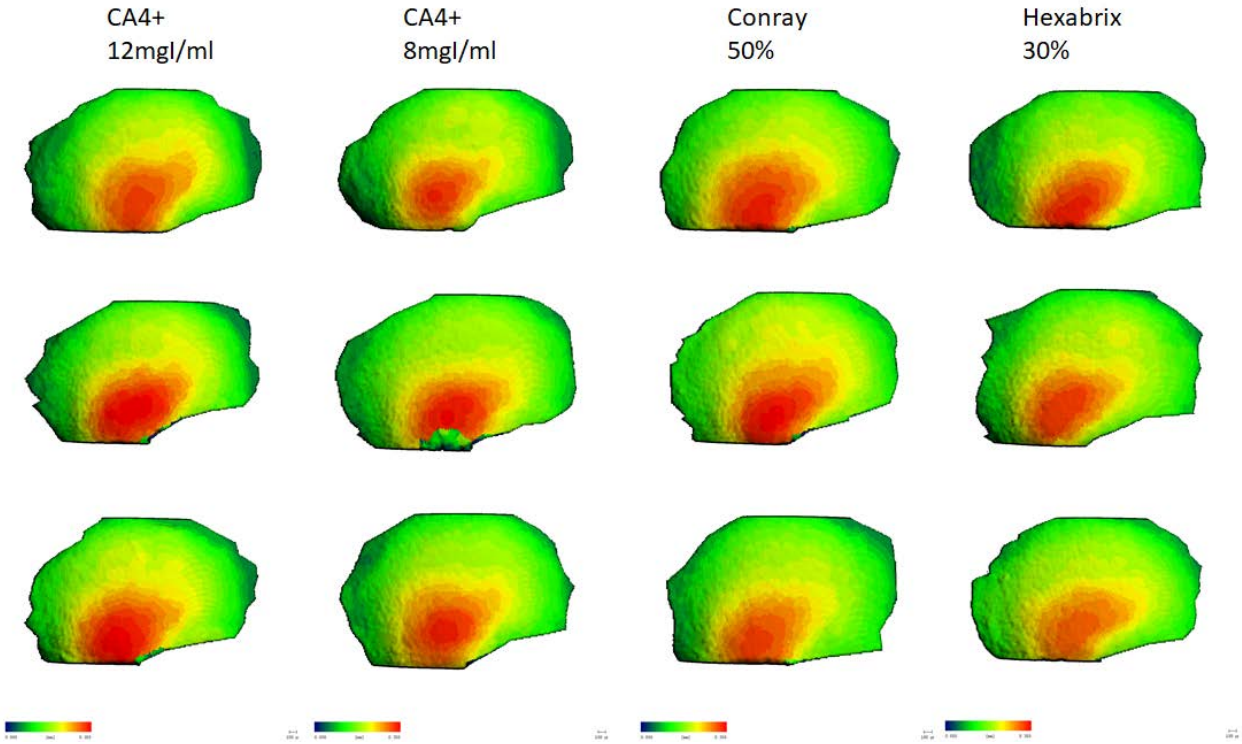

Figure 11. Medial tibial plateau articular cartilage thickness maps of right contralateral joints, 3 weeks post-surgery. Cartilage contrast was enhanced with CA4+ at two concentrations (8 and 12 mgI/ml), Conray™ at 50%, and Hexabrix™ at 30% (original protocol concentration).

Quantifications in previous studies using Hexabrix™ indicated changes (higher X-ray attenuation for MMT) were detectable in the medial and central thirds of the medial tibial plateau after 3 weeks. The following plots (Figure 12) show average X-ray attenuation in these regions.

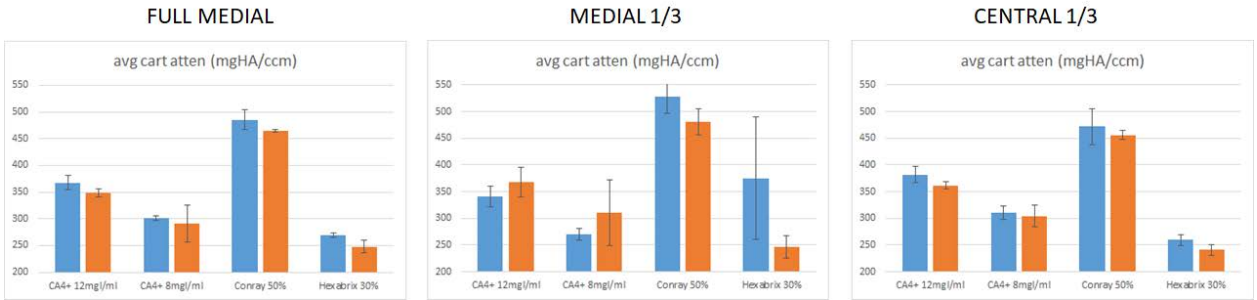

Figure 12. Quantification of average articular cartilage X-ray attenuation values in medial tibial plateau VOIs (full, medial third, central third). Blue = left MMT, orange = right contralateral (n=2-3, mean ± SD).

Though this pilot was not sufficiently powered for statistical findings, measurements made using CA4+ at 12 mgI/ml appeared less variable than that from the 8 mgI/ml concentration group. With this cationic contrast agent, it was expected that degenerated tissue would produce lower average X-ray attenuation values compared to healthy PG-rich tissues.

Average attenuation for medial third MMT tissue appeared to be lower than contralateral control, but this was not the case for full medial and central third measurements.

Nevertheless, CA4+ contrast enhancement with the 12 mgI/ml concentration provided three important characteristics: 1) low variability, 2) ability to visualize and quantify articular cartilage morphology parameters, 3) X-ray attenuation values in a range that should allow for discrimination of effects due to degeneration or therapeutics in a full study with the rat MMT model.

Additionally, Conray™ contrast enhancement demonstrated potentially useful characteristics: 1) relatively low variability, 2) equilibration / plateau in X-ray attenuation values after a relatively short immersion time instead of a continued (and slower) increase of X-ray attenuation as seen with CA4+, which is more similar to equilibration in Hexabrix™.

Initial tests using 12 mgI/ml CA4+ in ovine femoral groove tissue produced unexpected logistical challenges even after 2+ days of immersion time (uneven contrast enhancement in different cartilage regions of similar location and thickness, particularly intense X-ray attenuation values at the cartilage-subchondral bone interface). Because X-ray attenuation equilibration in Conray™ was deemed more stable, less sensitive to precise immersion timing, and faster, we decided to proceed with using Conray™ for further ovine tissue characterization for the full therapeutic efficacy study specimens.

Subtask 4: Ovine cartilage and subchondral bone characterization. (Completed and previously documented in Year 2 annual report).

Major Task 5: Assess therapeutic efficacy in ovine OA model.

Subtask 1: Surgical and treatment procedures.

Surgical procedures and subsequent dHACM injections for assessing efficacy of dHACM in the ovine MM model (single 24 hr, single 9 wk, multiple 24 hr + 9 wk) have been performed for all animals. All animals have been euthanized and stifle joints harvested and shipped from SAMMC to GT.

Experimental design was adjusted to include only 12 week total study durations because of two primary factors: 1) Animals at 12 wks post-MM exhibited qualitative damage

(cartilage, osteophytes) consistent with OA progression, 2) significant delays in CRADA and IACUC approvals shortened the timeframe in which the study could take place, and it was beneficial to reduce total animal numbers and study duration.

Additionally, due to results from Specific Aim 2 that suggested a 3 week therapeutic window for dHACM on degradative effects once OA progression has begun, the delayed injection timepoint was adjusted to 9 weeks post-MM.

The experimental design is shown in Table 1 below:

Table 1. Experimental design for the full ovine study included 4 therapeutic efficacy groups as described below (groups 1-4, n=6 per group), with a full study duration of 12 weeks. Additionally, stifle joints for animals in groups 5-7 were given dHACM injections 24 hrs post-MM (or after sham or no surgical procedure), and joints will be evaluated histologically to locate any dHACM presence or other inflammatory/cellular response after 1 week.

group	L stifle joint	R stifle joint	takedown	n
1	Sham	MM+24hr saline	12 wk	6
2	Sham	MM+24hr dHACM	12 wk	6
3	Sham	MM+24hr+9wk dHACM	12 wk	6
4	Sham	MM+9wk dHACM	12 wk	6
5	unoper+24hr dHACM	MM+24hr dHACM	1 wk	2
6	sham+24hr dHACM	MM+24hr dHACM	1 wk	1
7	unoper+24hr dHACM	unoper+24hr dHACM	1 wk	1

Subtask 2: Inflammatory response evaluation. See Specific Aim 1, Subtasks 2 and 4.

Subtask 3: Evaluation of therapeutic effects.

Initial tests with femoral groove tissue suggested that over 2 days of equilibration in 10-20% Conray™ solution would be effective for enhancement of cartilage tissues to an appropriate range for segmentation from surrounding air as well as subchondral bone in the articular cartilage regions of interest.

Using 15% Conray™ solution (80ml), proximal tibial and distal femoral condyle segments were individually immersed for 3 days then scanned with a Scanco µCT50 at 50µm voxel size. Axial 2D slice tomograms were reformatted to coronal slices, and articular cartilage segmented from air and bone using contouring and thresholding procedures.

Scans and evaluations for these specimens are ongoing. Images below are representative cartilage-bone overlays as well as bone only segmentations for distal femoral and proximal

tibial tissue from one animal from Group 1 (Left = sham, Right = MM+24hr saline injection, 12 weeks post-MM).

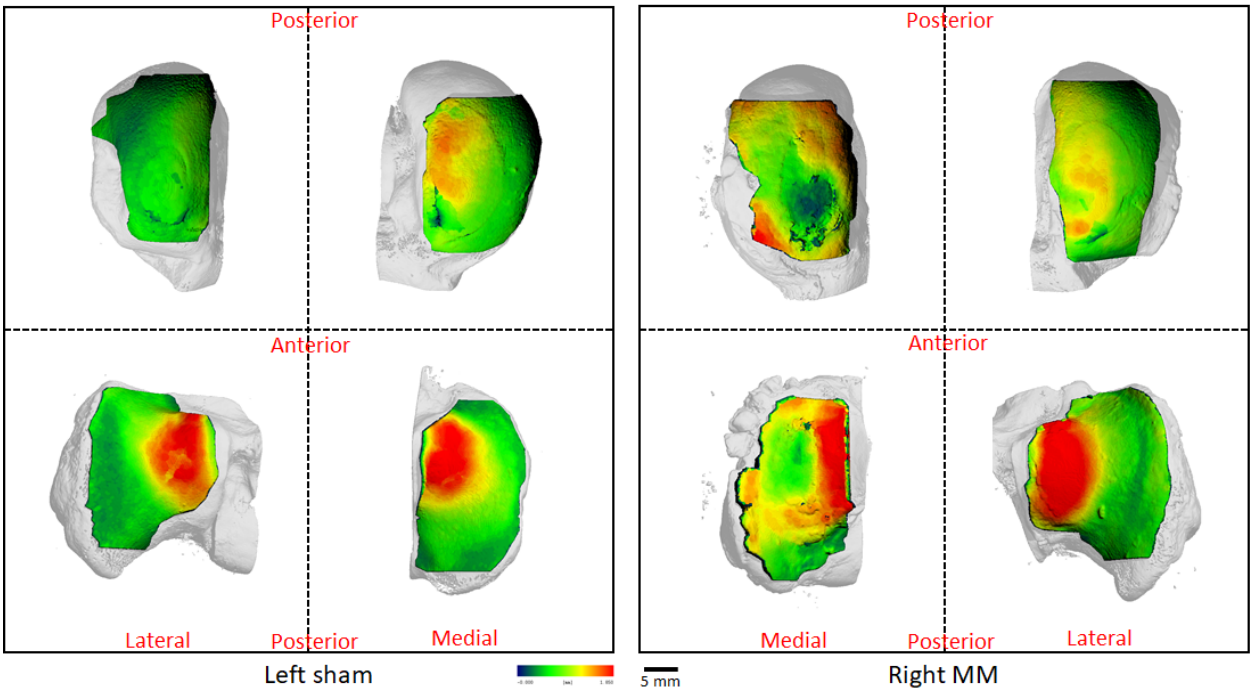

Figure 13. Representative articular cartilage-bone overlay images for Group 1; femoral condyles on the top row and tibial plateaus on the bottom row. Note the anatomical orientation markers in red text. Cartilage thickness maps are displayed (red = thicker, blue/green = thinner). Right stifle joint underwent MM with 24hr post-MM saline injection; Left = sham contralateral. Visible damage can be seen in the right MM joint, particularly on the medial femoral condyle and medial tibial plateau. The right MM joint also appears to have thickened areas of cartilage on the lateral femoral condyle and lateral tibial plateau compared to the left sham joint.

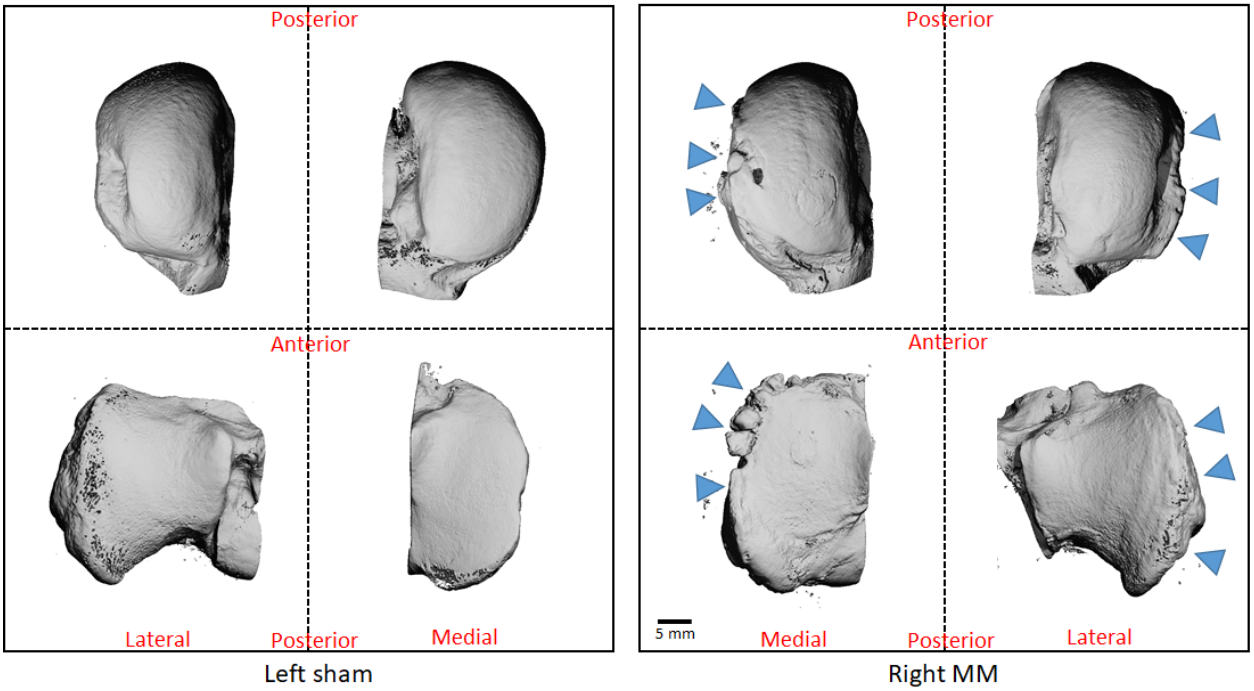

Figure 14. Representative bone only segmentations for the same animal from Figure 13 (Group 1); femoral condyles on the top row and tibial plateaus on the bottom row. Note the anatomical orientation markers in red text. Likely marginal osteophyte formations are visible in the right MM joint, as indicated with blue arrows.

These images suggest that at 12 wks post-MM surgery, with a saline control injection at 24 hrs post-MM, there are observable degenerative changes in the affected knee tissues. Imaging of the remaining joints and further quantitative analyses of articular cartilage, subchondral bone, and osteophyte characteristics continue.

Specific Aim 3 Conclusions

In the Year 2 annual report, pilot study imaging suggested that the MM surgical technique in ovine stifle joints induced detectable degenerative changes at 12 weeks post-surgery. The experimental design for the full therapeutic efficacy study was adjusted accordingly.

Work was conducted in Year 3 to determine a suitable alternative contrast agent, as the original contrast agent was discontinued. After characterization of a cationic and an anionic contrast agent in rat and ovine tissue, it was deemed best for the ongoing ovine tissue work to utilize the anionic contrast agent, Conray^{TM,}, at 15% concentration for an immersion time of 3 days at room temperature.

Preliminary imaging of joints injected with saline at 24 hrs post-MM after 12 wks were consistent with pilot study imaging results. Work in the coming year will include completing the imaging processes as well as running full quantifications of articular cartilage, subchondral bone, and osteophytes using microCT as well as histopathology.

Current and Anticipated Problems

There have been a few issues that have resulted in adjusted study timelines and experimental procedures, as listed below:

1. *Inflammatory response evaluation in the rat MMT model*: (Specific Aim 1, Major Task 2, Subtask 2; Specific Aim 2, Major Task 3, Subtask 2); Synovial cellular response: (Specific Aim 1, Major Task 2, Subtask 4; Specific Aim 2, Major Task 3, Subtask 4)

The originally proposed methodology was to extract synovial fluid from rat joints via knee joint lavage technique. However, this technique proved to be inconsistent in acquiring enough fluid to analyze. The alternative approach we utilized was to better understand how the dHACM particles, which we have shown become embedded in the synovial membrane in the rat MMT model, interact with synoviocytes and chondrocytes in vitro using Transwell culture.

2. *Assessing therapeutic efficacy of dHACM treatment in ovine MM model*: (Specific Aim 3, Major Task 5)

There are two major issues that have affected the progress of this portion of the project:

IACUC approval for the therapeutic efficacy study in the ovine MM model was extremely delayed. Release of funding for the subaward to SAMMC occurred in Year 3. The animal work portion of the study has been completed, but the imaging and analyses portions have been substantially delayed.

The originally utilized cartilage contrast agent, Hexabrix™, was discontinued from production by its manufacturer. Testing of a cationic contrast agent called CA4+ and an anionic contrast agent called Conray™ was performed in Year 3 (in healthy and diseased joints of rats as well as ovine joint tissue) for these applications. Conray™ will be used moving forward for this portion of the project. However, the testing and validation processes were time consuming, thus creating delays in the imaging and analyses.

4. KEY RESEARCH ACCOMPLISHMENTS:

1. We determined through work in Specific Aim 2 that intra-articular treatment with dHACM after the onset of OA changes can provide protective effects against disease progression (evaluation of effects at 6 weeks post-MMT surgery when dHACM treatment was administered at 3 wks post-surgery).

2. Evaluation of disease progression at 1 and 3 weeks post-MMT surgery in the rat model (from Specific Aim 1) suggested that degenerative changes in articular cartilage (attenuation and surface roughness) were detectable prior to those of subchondral bone (mineral density and thickness). This addresses a major question in the field on the sequential pathology of post-traumatic OA.

3. Reduction of the particle size of micronized dHACM did not further attenuate OA progression in the rat MMT model. Notably, 24 hr post-MMT treatment with standard particle size dHACM provided better protection against proteoglycan loss after 1 and 3 weeks, and 24 hr post-MMT treatment with reduced particle size dHACM increased cartilage thickness and lesion volume after 3 weeks. Particle retention in the joint as quantified by tagging of the particles and fluorescent efficiency measured over time suggested that there was no difference in retention for the standard dHACM particle size compared to the reduced particle size dHACM. Thus, the difference in therapeutic effects was likely not due to particle retention time in the joints.

4. Testing of cationic contrast agent CA4+ and anionic contrast agent Conray™ as suitable replacements for anionic contrast agent Hexabrix™ was performed using rat and ovine tissues. Both of these contrast agents have potential advantages. For further ovine tissue characterization in the dHACM therapeutic efficacy study, Conray™ will be utilized.

5. CONCLUSION:

The combined effects of post-traumatic and degenerative OA on active military personnel and veterans are the cause of substantial disability, medical discharge, and long-term costs. Assuming success of this work and further clinical studies to demonstrate safety and effectiveness of the proposed treatment method, the injectable nature of the therapeutic could facilitate accessibility of treatment in the field. Because OA disease progression is accelerated for soldiers who experience high energy combat injuries, one potential course of action would be to administer the therapeutic immediately post-operatively in an effort to prevent the accelerated response and reduce the loss of personnel and costs associated with disability.

An important consideration for injectable OA therapeutics is the ability to be retained in the joint and therefore sustain beneficial effects and minimize the frequency of injections. This work will provide necessary preclinical data including safety and immune response, retention, and long-term effectiveness of the particle injections. If successful, this project will present a readily translatable, low risk, minimally invasive treatment method that modifies OA disease progression in a clinically relevant model, leading to human clinical trials as the clear next step.

Much progress has been made thus far on the study aims. For the small animal studies, two particle size distributions of dHACM were assessed for their therapeutic effects after MMT, and the standard micronized formulation of dHACM (AmnioFix®) resulted in better preservation of articular cartilage PG content after 1 and 3 weeks. Reducing the particle size (RPS AmnioFix®) did not further attenuate OA disease progression compared to the standard formulation and resulted in higher average cartilage thickness and higher lesion volumes at 3 weeks. Surface roughness was utilized effectively as a measure for cartilage degenerative changes at 1 and 3 weeks post-MMT. Effects of single early, single delayed, and multiple dHACM intra-articular treatments were assessed in the rat MMT model, and the single delayed injection (emulating treatment after disease progression has begun) provided a protective effect

against degradative changes in the joint. dHACM particles have been successfully labeled with a fluorophore, and measured retention profiles of two particle size distributions in the rat MMT model did not differ. Based on in vitro studies with synoviocytes and synoviocyte/chondrocyte co-cultures, dHACM modulated inflammatory cytokine activity and protected chondrocyte pellets from proteoglycan loss induced by IL-1β, TNF-α, and synoviocytes.

One critical focus area that remains to be completed is assessing the effects of dHACM in the ovine MM model of PTOA. Contrast enhanced imaging and characterization of the joints from this full therapeutic efficacy study is ongoing. Once joint tissues are evaluated with microCT techniques at GT, they will be returned to SAMMC for histopathology analyses. Due to the aforementioned problems and delays, we requested a 12 month no cost extension to complete the remaining work on this portion of the project.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Publications:

Status	Description
Accepted	Reece, D.S., Thote, T., Lin, A.S.P., Willett, N.J., Guldberg, R.E. Contrast Enhanced MicroCT Imaging of Early Articular Changes in a Pre-Clinical Model of Osteoarthritis," Osteoarthritis & Cartilage, accepted October 2017.
Submitted	Thote, T., Reece, D.S., Sridaran, S., Lin, A.S.P., Stevens, H.Y., Willett, N.J., Guldberg, R.E. "Intra-Articular Delivery of Micronized Dehydrated Human Amnion/Chorion Membrane Reduced Degenerative Changes in Osteoarthritic Joints," Arthritis Research & Therapy, submitted October 2017.

Presentations:

Type	Description
Conference presentation / poster	Reece, D.S., Mancipe Castro L.M., Salazar-Noratto, G.E., Burnsed, O.A., Lin, A., Guldberg, R.E. Influence of Particle Size on Micronized Amnion/Chorion Membrane Therapeutic Efficacy in a Post-Traumatic Osteoarthritis Rat Model," March 2017.

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report.

8. REPORTABLE OUTCOMES: These studies have resulted in creation of a research tool based on microCT scans to measure two novel 3D metrics for joint degeneration: surface roughness (based on differences with model surfaces) and exposed bone area. It has been tested and validated for our preclinical rat model of PTOA and will be tested and validated in the ovine model. This algorithm may provide benefit in the investigation of early effects of potential therapeutics. A manuscript, entitled "Contrast Enhanced MicroCT Imaging of Early Articular Changes in a Pre-Clinical Model of Osteoarthritis," has been accepted for

publication in Osteoarthritis & Cartilage. It includes supplemental documentation for utilizing this research tool, and we have made the MATLAB program available via download at this location: http://guldberglab.gatech.edu/downloads.

9. **OTHER ACHIEVEMENTS:** David S. Reece, a graduate student who had been conducting work supported by this grant, earned a PhD at the Georgia Institute of Technology during the Year 3 funding period.